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CHARACTERIZATION BY NMR AND FLUORESCENCE SPECTROSCOPY OF DIFFERENCES IN THE CONFORMATION OF NON-AGED AND AGED ORGANOPHOSPHORYL CONJUGATES OF AChE

Annual Summary Report

Y. ASHANI[1]
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September 10, 1984

Supported by

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The objective of this project is to characterize, by nmr and by fluorescence spectroscopy, differences in the conformations of non-aged and aged organophosphoryl conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht). In the present study, O-1-pyrenebutyl O-ethylphosphorofluoridate (PBEPF) and O-1-pyrenebutyl phosphorodichloridate (PBPDC) were used to obtain the non-aged and aged conjugates of Cht respectively. ^{31}P-nmr spectroscopy and reactivation studies support the hypothesis that the aged form, pyrenebutyl-O-P(O)(OH)Cht, contains a P-O(-) function.

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FIGURE 2

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Significant conformational differences between the aged conjugate and the non-aged conjugate, pyrenebutyl-O-P(O)$(OC_2^{\pi}H_5^{\pi})$Cht, were indicated by the optical spectroscopic results (steady-state and time-resolved fluorescence, circular dichroism (CD) and circularly polarized luminiscence (CPL) measurements). The interaction of the fluorophore with the protein backbone appears to be stronger for the aged conjugate than for the non-aged phosphorylated enzyme. Two conclusions have been derived so far:

a) The dihalide organophosphates employed are suitable for preparing aged organophosphoryl conjugates of Cht (and presumably of other serine hydrolases) which can be compared with the corresponding non-aged conjugates.

b) The spectroscopic data provide a partial interpretation for the known resistance of aged conjugates to reactivation.

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SUMMARY

The objective of this project is to characterize, by nmr and by fluorescence speotroscopy, differences in the conformations of non-aged and aged organophosphoryl conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht). In the present study, O-1-pyrenebutyl O-ethylphosphorofluoridate (PBEPF) and O-1-pyrenebutyl phosphorodichloridate (PBPDC) were used to obtain, respectively, the non-aged and aged conjugates of Cht. ^{31}P-nmr spectroscopy and reactivation studies support the hypothesis that the aged form, pyrenebutyl-O-P(O)(OH)Cht, contains a P-O$^-$ function. Significant conformational differences between the aged conjugate and the non-aged conjugate, pyrenebutyl-O-P(O)(OC_2H_5)Cht, were indicated by the optical spectroscopic results (steady-state and time-resolved fluorescence, circular dichroism (CD) and circularly polarized luminescence (CPL) measurements). The interaction of the fluorophore with the protein backbone appears to be stronger for the aged conjugate than for the non-aged phosphorylated enzyme. Two conclusions have been derived so far:

a. The dihalide organosphosphates employed are suitable for preparing aged organophosphoryl conjugates of Cht (and presumably of other serine hydrolases) which can be compared with the corresponding non-aged conjugates.

b. The spectroscopic data provide a partial interpretation for the known resistance of aged conjugates to reactivation.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

This work has been conducted with the collaboration of: G. Amitai, A.C.M. Van Der Drift, J. Grunwald, E. Haas, Y. Segall, N. Steinberg and D. Wysbort.

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OBJECTIVES

1. General

The objective of this study is to characterize by nmr and fluorescence spectroscopy differences in the conformation of non-aged and aged organophosphoryl (OP) conjugates of acetylcholinesterase (AChE) and chymotrypsin (Cht), which might help to explain the unexpected resistance of the aged forms to commonly used reactivators.

2. Studies with Cht

During the period covered by this report (September 1983-August 1984) the principal lines of research were as follows:

a. Preparation of stoichiometric fluorescent and non-fluorescent aged and non-aged OP conjugates of Cht.

b. Characterization of the fluorescent and non-fluorescent OP conjugates by nmr spectroscopy.

c. Characterization of the pyrene-containing OP conjugates of Cht by fluorescence spectroscopy.

It was assumed that the nmr spectroscopy of the OP-Cht conjugates would permit us to elucidate the substituents attached to the P atom in the aged and non-aged conjugates produced by the di- and monohalides, respectively. Furthermore, optical spectroscopy would permit us to detect putative conformational differences between the two conjugates which might explain the resistance of the aged conjugate to reactivation relative to the corresponding non-aged conjugate (for details, see Technical Approach section).

BACKGROUND

In our original proposal, we envisaged studying the aging process in OP conjugates of Cht in parallel to OP conjugates of AChE, since Cht is a well-characterized enzyme whose sequence and 3-dimensional structure have been fully worked out. Furthermore, it is commercially available in highly purified form in large quantities, allowing preparation of OP-Cht conjugates at millimolar concentrations. This permits not only fluorescence studies but also ^{31}P-nmr spectroscopy. Such data can thus be meaningfully correlated with the structural data, hopefully leading to detailed understanding of the aging process at the molecular level.

Irrespective of the mechanism of aging, it is usually accepted that the common denominator is the net loss of an alkyl or alkoxy group from the enzyme-bound OP moiety. This loss is depicted in scheme I. This

scheme implies that aging is associated with the introduction of a formal negative charge into the active site of the inhibited enzyme.

$$EH + XP(O)R(OR') \xrightarrow{-HX} \underset{\text{inhibited enzyme}}{E\text{-}P(O)R(OR')} \xrightarrow{-R'} \underset{\text{aged enzyme}}{E\text{-}P(O)R(O^-)}$$

where: R = alkyl, aryl, alkyloxy or aryloxy
R'= alkyl, aryl
X = F^-, Cl^-, p-nitrophenol, dialkylaminoethanethiol.

Scheme I

The observation that phosphoric ester dihalides of the general formula $(RO)POCl_2$ immediately produce a non-reactivatable enzyme and therefore presumably an aged enzyme (1,2) substantiates the hypothesis that aging involves formation of a negatively charged oxygen atom at the active site.

Nevertheless, no direct evidence was reported, until the activation of this project in 1983, to confirm the presence of $P\text{-}O^-$ in the active site of the aged form of the OP conjugate of any serine hydrolase. Only recently, Van der Drift (3) demonstrated, by comparative ^{31}P-nmr spectroscopy, the presence of $P\text{-}O^-$ in the aged conjugate of Cht that had been inhibited by diisopropylfluorophosphate (DFP).

In view of the above, it was envisaged that by performing nmr spectroscopy in parallel to fluorescence measurements of the same batch of the OP-Cht conjugates, we might achieve the following:

a. Substantiate the hypothesis that phosphoric acid derivatives of mono- and dihalide esters should provide the corresponding non-aged and aged conjugates, respectively.

b. Permit a parallel study of OP conjugates of Cht and AChE with respect to the aging process, by employing the same alkyl halide phosphates for the inhibition of both enzymes.

c. Provide direct evidence concerning the nature of the substituents attached to the P atom in the non-aged and aged conjugates of Cht obtained by employing alkyl halide phosphate inhibitors.

d. Provide evidence by optical spectroscopy for putative conformational differences between aged and non-aged fluorescent OP conjugates of Cht, which might explain the resistance of the aged conjugates to reactivation.

e. Validate the applicability of mono- and dihalide phosphates as a general preparative approach for the introduction of different

fluorophores in or near the active site of non-aged and aged conjugates of AChE.

Fluorescent probes are used to investigate parameters such as environment, conformation, shape, rotational freedom and proximity of sites on macromolecules. In our studies we decided to use pyrene as the fluorescent probe (see Fig. 1 for detailed structure). This fluorophore has proved valuable due to its excitation wavelength being well-separated from the protein absorption band, due to its high quantum yield and its long radiative lifetime (4). In our comparative studies on aged and non-aged conjugates of AChE containing this fluorophore, we were able to take advantage of these properties in several ways.

a. We have utilized fluorescence decay measurements so as to show that differences in relative quantum yield between aged and non-aged conjugates may be ascribed to dynamic quenching (2).

b. We have been able, using time-resolved anisotropy, to show that there is little overall difference in the shape of the protein molecule in the aged and non-aged conjugates, although the peripheral site ligand propidium may induce shape changes in both conjugates (unpublished results).

c. Utilizing circularly polarized luminescence, we have been able to clearly demonstrate a change in the environment of the pyrene in the active site of both aged and non-aged conjugates induced by propidium (5).

d. Using collisional quenching, by external quenching agents, we have been able to demonstrate reduced accessibility of the pyrene chromophore to an external quencher in the aged conjugates relative to the non-aged conjugates (2).

Thus, in view of the above, we used compounds 1-3 (see below) to obtain the aged and non-aged OP conjugates of α-Cht containing the pyrene fluorophore.

TECHNICAL APPROACH

1. Preparation of conjugates

In order to obtain the non-aged and aged analogues of Cht, we studied the kinetics of inhibition of Cht with the following organophosphates, and then prepared the corresponding purified stoichiometric conjugates:

RO-P(O)(Y)(X)

cpd	name	R	Y	X	Source or method of preparation
1	PBEPF	Pyrenebutyl	OC_2H_5	F	(2)
2	PBEPC	Pyrenebutyl	OC_2H_5	Cl	(2)
3	PBPDC	Pyrenebutyl	Cl	Cl	(2)
4	Paraoxon	C_2H_5	OC_2H_5	p-nitrophenol	Aldrich (used as obtained)
5	DEPC	C_2H_5	OC_2H_5	Cl	Aldrich (redistilled)
6	DEPF	C_2H_5	OC_2H_5	F	(2)
7	EPDC	C_2H_5	Cl	Cl	Aldrich (redistilled)

Compounds 1-7 were found to contain only one kind of phosphorus moiety as could be judged from ^{31}P-nmr spectroscopy. Compounds 1, 2, 4, 5 and 6 were assumed to yield the non-aged conjugates, whereas 3 and 7 were expected by analogy with AChE(2) to instantaneously generate the corresponding aged form of Cht.

Large quantities of the OP-Cht conjugates were obtained by adding, dropwise, a concentrated solution of the inhibitor (0.01 - 0.1 M) in an organic solvent (e.g. acetonitrile, acetone) to a stirred solution of 5-10 mg/ml α-Cht (Sigma, bovine pancreas, 3X crystalized) in double distilled water. 400 mg of α-Cht were inhibited in each experiment. The decrease in activity was monitored to completion by employing the pH-stat technique of Goldstein (6). In the case of compounds 1-3, the inhibition was carried out in dilute solutions of both α-Cht (0.5 - 1.0 mg/ml) and the inhibitors (0.2 - 1 mM) because of the limited solubility of the pyrene OP ligands in water. The amount of the organic solvent in the final inhibition medium did not exceed 10%. The OP-Cht conjugate was lyophilized, redissolved in water, separated from free ligand by

chromatography on a Sephadex G-10 column and relyophilized. Occasionally, samples were dialyzed for 24 hr at 4°C so as to remove traces of hydrolysed free inhibitor which were detected by nmr spectroscopy.

For conjugates obtained from compounds 1-3, the ratio of absorptions at 344 and 280 nm indicated approximately 1:1 (±15%) stoichiometry of organophosphate and protein in the conjugate. The specificity of binding of 1-3 to Cht was demonstrated by preincubating the enzyme with paraoxon before treatment with the pyrenebutyl organophosphates. The fluorescence of the paraoxon-treated Cht was found to be less than 5% of that of the non-treated samples. The OP-Cht conjugates were then studied by nmr and fluorescence spectroscopy.

2. nmr measurements

^{31}P-nmr spectroscopy at 101.3 MHz was performed with a Bruker WM250 spectrometer coupled to a Nicolet Aspect 2000 computer. 20-99% D_2O in the sample tube (10 mm diameter) served as an internal standard for field frequency locking. A power of 10 watts was maintained for the continuous broadband heteronuclear proton decoupling so as to avoid internal build-up of heat. Throughout the run the temperature was maintained at 23±3°C. Spectral data were accumulated in the Fourier transform by application of 70° pulses with a spectral width of 16000 Hz. A delay time of 0.8 sec was utilized between accumulations.

All chemical shifts were recorded from the built-in absolute crystal frequency and assigned to the internal standard, N,N-hexamethylphosphorotriamidate (HMPA). The ^{31}P-nmr spectra of the Cht conjugates and the model compounds were recorded in a concentration range of 1-10 mM. 5-100K scans were accumulated for each run.

3. optical spectroscopy

UV absorption spectra were measured in a Cary 219 spectrophotometer. Circular dichroism (CD) spectra were recorded using a Cary 60 apparatus at 26°C. The spectra are expressed in terms of the absorption anisotropy factor, g_{ab}, which is defined as $g_{ab} = \Delta\varepsilon / \bar{\varepsilon}$ where $\Delta\varepsilon$ is the difference between the molar extinction coefficients for left- and right-handed circularly polarized light, and $\bar{\varepsilon}$ is their average.

Fluorescence decay measurements were performed with an instrument built at the Weizmann Institute which employs the sampling technique of Hazan et al., (7). Decay curves were analyzed by the method of non-linear least squares (8) assuming fluorescence decay according to $I(t) = \sum_i \alpha_i e^{-t/\tau_i}$, where I(t) is the decay function and α_i and τ_i are, respectively, the amplitude and lifetime of the i^{th} component.

Measurements of circular polarization of luminescence (CPL) were performed with an instrument built at the Weizmann Institute (9). CPL relates to the molecular conformation of the chromophore in its emitting excited state in the same way that CD relates to the molecular conforma-

tion in the ground state. Thus, the anisotropy factor of emission, g_{em} is defined as $g_{em} = \Delta I/(I/2)$, where ΔI is the intensity of the circularly polarized component in the emission, and I is the total luminescence intensity.

Unless stated otherwise, all measurements were performed at room temperature ($23 \pm 1^{\circ}C$). Protein concentrations were in the range of 5×10^{-7} M (fluorescence) to 5×10^{-5} M (CD, CPL).

RESULTS AND DISCUSSION

1. nmr spectroscopy

N,N-Hexamethylphosphorotriamidate $[(CH_3)_2N]_3P(O)$, HMPA, was used as an internal standard throughout this study.[3] The chemical shift of HMPA is 30.50 ppm downfield to external 85% phosphoric acid and is well separated from the signals of the model compounds and the protein-OP conjugates (Fig. 1). The mean value of the chemical shift of HMPA, as recorded from the built-in absolute crystal frequency, was found to be 8919±3 (n=60) Hz (or 88.05± 0.03 ppm), whereas 85% phosphoric acid absorbs at 57.55± 0.03 ppm as related to the same crystal frequency.

The results obtained for HMPA were recorded in 60 different experiments where the pH ranged between 2 and 9 and the solution contained either 1-5 mM OP-Cht conjugate or 1-10 mM of the model compound. Thus, the chemical shift of HMPA was not sensitive to these variations, and remained constant throughout. However, in several cases we observed that, in the presence of 6 M guanidine hydrochloride, a very small upfield shift occurred (8900±5 Hz), relative to the other measurements.

In order to correlate ^{31}P chemical shifts of the aged and non-aged conjugates of Cht with the various substituents attached to the P atom, we first established the ^{31}P chemical shifts of the model compounds under the same experimental conditions that were used for the OP-Cht conjugates. Table 1 summarizes the results of these experiments.

Table 1: ^{31}P-nmr Chemical Shifts of Model Compounds

Type	Structure	δ, ppm * relative to HMPA	pH
Tertiary esters	$(C_2H_5O)_3PO$	-30.38	3-7
Secondary esters (mono acids)	Pyr-P(O)(OC_2H_5)(OH)	-29.42	3-7
	$(C_2H_5O)_2P(O)(OH)$	-29.19	
Primary esters (diacids)	Pyr-P(O)$(OH)_2$	-25.50	>8
	$(C_2H_5O)P(O)(OH)_2$	-26.15	>8

* Negative sign indicates upfield shift relative to HMPA.
Error estimate: <0.03 ppm; Pyr = pyrenebutyl-O-

Results are average of at least three measurements of different preparations.

The addition of 1-5 mM free or inhibited Cht did not affect the chemical shifts of the model compounds. The results in Table 1 show that replacing one alkoxy group in the phosphate triester molecule with a negatively charged oxygen, ao as to form a diester, causes a significant downfield ahift in the ^{31}P-nmr signal relative to the triester model compound. Similar shifts ($\Delta\delta$ 2.2. ppm) were alao observed by Van der Drift (3) with respect to diisopropylphosphates. The chemical shift is further displaced downfield upon insertion of a second ionized oxygen (diester→monoester). The pH-chemical shift profiles for $(C_2H_5O)_2P(O)OH$ and $(C_2H_5O)P(O)(OH)_2$ are depicted in Fig. 2. The titration curves show a downfield shift with increasing pH for both compounds. The correaponding pKa values, estimated from the pH-chemical shift titration curves, are as follows:

	pKa_1	pKa_2
$(C_2H_5O)_2P(O)OH$	1-2	-
$(C_2H_5O)P(O)(OH)_2$	1-2	6.5

These values are in good agreement with thoae reported in the literature for similar phosphates (10). Although the interpretation of the chemical shifts reported in Table 1 is complex in terms of steric and electronic contributions of the substituents to the degree of bonding to the P atom, it is clear from the model compounds reported here, taken together with the results obtained by Van der Drift (3), that changing the structure of the triester to the diester, $(RO)_3P(O)$→$(RO)_2P(O)OH$), leads to a shift of $\Delta\delta$ 1-2 ppm downfield from the triester.

Table 2 summarizes the chemical shifts observed for the various OP-Cht conjugatea. The relative chemical shifts of the phosphorylated non-aged (assuming a triester structure) and aged Cht (assuming a diester structure) were found to be as predicted from the corresponding ^{31}P-nmr chemical shifta of the model compounds (Table 1). Thus, the ^{31}P-nmr signals of the non-aged forms appeared $\sim$ 1 ppm upfield to the aged conjugates in the native preparations. After denaturation, the chemical shifts for both aged and non-aged conjugates moved upfield and became close to the reported values for the tri- and diester model compounds, thus maintaining the relative differences in chemical shift observed prior to denaturation. The consistency of the downfield shift of the various diesters relative to the corresponding triesters is shown in Table 3. A similar downfield shift, but somewhat larger ($\Delta\delta$, 2-2.5 ppm), was observed by Van der Drift (3) for the corresponding diisopropylphosphoryl conjugates of Cht.

A pH-chemical shift dependence profile was observed for the native diethylphosphoryl-Cht conjugate. The upfield shift in the ^{31}P-nmr signal with increasing pH (Fig. 3) displayed a titration curve with one titratable group (pKa $\sim$ 7.1). Since the pH-chemical shift for the protein conjugate was in the opposite direction from the titration curve obtained for both the dieater and the monoester model compounds (Fig. 2), we may conclude that the profile depicted in Fig. 3 reflects a

Table 2: ^{31}P Chemical Shifts (δ, ppm relative to HMPA) of Native and Denatured OP-Cht Conjugatea.

Type	Structure	δ, ppm[e] (from HMPA)	pH
Trieaters	(N)Pyr-P(O)(OC_2H_5)Cht[a]	-28.62	3-7.5
	(D)Pyr-P(O)(OC_2H_5)Cht[b]	-30.97	3-7
	(N)$(C_2H_5O)_2$P(O)Cht[a]	-28.53	3-5
	(D)$(C_2H_5O)_2$P(O)Cht[c]	-31.02	3-8
Diesters	(N)Pyr-P(O)(OH)Cht[a]	-27.54	2-9
	(D)Pyr-P(O)(OH)Cht[b]	-29.28	3-7
	(N)(C_2H_5O)P(O)(OH)Cht[a]	-27.60	3-5
	(D)(C_2H_5O)P(O)(OH)Cht[b]	-29.28[d]	4.2

a) N = native; Pyr = pyrenebutyl-O-
b) D = denatured with 6 M guanidine hydrochloride
c) assumed to have undergone denaturation during preparation of the conjugate
d) single measurement
e) estimated errors ≤0.03 ppm

Results are average of at least three measurements of preparations obtained on different occasions.

change in interaction of the phosphorus ligand with the protein backbone and not dissociation of a P-OH group. The pK_a calculated for the single titratable acidic group is in good agreement with the value reported for the diisopropylphosphoryl-Cht conjugate (3). We note that the same pH-chemical shift profile was obtained for conjugates derived from either DEPF or paraoxon, thus substantiating the assumed structure, $(C_2H_5O)_2P(O)Cht$.

The denatured diethylphosphoryl conjugate did not show such a pH-chemical shift profile, presumably because denaturation diminished interaction of the phosphoryl moiety with the protein backbone. More experiments will be required in order to establish whether such a pH-chemical shift dependence exists for the non-aged pyrenebutyl OP-Cht conjugate, since initial experiments revealed only a small shift. No pH-chemical shift dependence was detected for any of the aged conjugates, whether nature or denatured.

Several reactivation experiments were conducted in order to correlate resistance to reactivation of the aged conjugate with the

Table 3: Δδ, ppm for various sets of trieaters and diesters

Structure	Protein	Δδ, ppm (δtriester-δdiester)
Pyr[b]-P(O)(OC_2H_5)Cht Pyr-P(O)(OH)Cht	native	1.08
Pyr-P(O)(OC_2H_5)Cht Pyr-P(O)(OH)Cht	denatured	1.69
$(C_2H_5O)_2$P(O)Cht (C_2H_5O)P(O)(OH)Cht	native	0.93
$(C_2H_5O)_2$P(O)Cht (C_2H_5O)P(O)(OH)Cht	denatured	1.74
$(C_2H_5O)_3$PO $(C_2H_5O)_2$P(O)(OH)	-	1.26
$(C_2H_5O)_3$PO Pyr-P(O)(OC_2H_5)(OH)	-	0.96

a) indicates downfield ahift relative to the triester; error estimate >0.03 ppm

b) Pyr = pyrenebutyl-O-

presence of the P-O^- group. Indeed, in the presence of 0.1 M 3-pyridinealdoxime methiodide (3-PAM), 56% of the activity of the non-aged conjugate, pyrenebutyl-OP(O)(OC_2H_5)Cht, was restored within 43 hr at 25°C, whereas no reactivation was observed for the similarly treated aged conjugate, pyrenebutyl-OP(O)(OH)Cht. Table 4 summarizes the results of this experiment. The ^{31}P-nmr spectroscopy of the reactivation process is currently under investigation. We have noted, in relatively old preparations of the non-aged conjugate, pyrenebutyl-OP(O)(OC_2H_5)Cht, the appearance of a ^{31}P signal which could be assigned to the model compound pyrenebutyl-OP(O)(OC_2H_5)OH. The appearance of

this signal (δ -29.4 ppm) was accompanied by the reappearance of a small but significant amount of enzyme activity, presumably due to spontaneous reactivation.

Table 4: Reactivation of pyrenebutyl-OP(O)(OC_2H_5)Cht and pyrenebutyl-OP(O)(OH)Cht (both 0.04 mM) in presence of 0.1 M 3-PAM (25°C, pH 7.0). (Single experiment)

Time (hr)	% activity	
	aged	non-aged
0	< 3	< 3
2	< 3	15
20	< 3	38
43	< 3	56
68	< 3	62.5

The ^{31}P-nmr spectroscopy results, along with the reactivation data, strongly support the hypothesis that the aged form of phosphorylated Cht contains a P-O bond and that the corresponding dihalides are suitable inhibitors for producing the inhibited aged conjugate.

2. optical spectroscopy

The aim of the optical spectroscopic investigations was two-fold: 1) to obtain spectroscopic evidence, in addition to the nmr data, that inhibition of Cht by either PBEPF or PBPDC yields different conjugates which can be distinguished from each other by the various optical techniques employed; 2) to find an explanation for the spectral differences in terms of differences in interaction of the OP moiety with the protein backbone.

Comparison of the UV absorption spectra (not shown) of denatured pyrenebutyl-OP(O)(OC_2H_5)Cht and Pyrenebutyl-OP(O)(OH)Cht with those of pyrenebutanol and of denatured Cht shows that in both cases there is a 1:1 stoichiometry (±15%) between the enzyme and the covalently attached pyrene moiety. The positions of the maxima in the UV-absorption spectra of the native conjugates are the same, but there are small differences in intensity in the range 250-310 nm.

The CD spectra (Fig. 4) reveal, in both conjugates, a marked induced asymmetyry of the pyrene chromophore, imposed by the tertiary structure of the enzyme molecule. This optical activity results from non-covalent interactions between the enzyme and the chromophore, since neither the conjugates denatured in 6 M guanidine hydrochloride nor the free pyrenebutanol show any optical activity i.e., g_{ab} is zero within experimental error. The anisotropy factors as obtained from the CD spectra are markedly different for the two undenatured conjugates, especially at a wavelength (ca. 350 nm) close to the peak of the absorption band of the pyrene group (Fig. 4). Thus, interaction of the pyrene moiety with the protein backbone is markedly different in the two conjugates.

The fluorescence emmission spectra of both conjugates show the same profile with two maxima at essentially the same wavelength (377 and 397 nm). However, the quantum yield of the non-aged conjugate is about 25% higher than that of the aged conjugate. This difference in quantum yield was confirmed by time-resolved fluorescence spectroscopy (Fig. 5). For both conjugates a good fit for the decay function could be obtained by assuming a two-component decay mechanism, with one component of long lifetime (ca. 100 nsec), and one of short lifetime (ca. 10 nsec). While the shorter component has about the same value (10-12 nsec) for the two conjugates, the longer lifetime is markedly different, oa. 105 nsec for the non-aged and ca. 80 nsec for the aged conjugate (Table 5). This difference can indeed account for the observed differences in quantum yield. Removal of oxygen by bubbling argon through the solutions does not diminish this difference, eventhough it leads to a small increase (ca. 5%) in the longer component for both conjugates. Thus the difference in quantum yield points to a different interaction between the pyrene moiety and the protein backbone in the two conjugates. This is further confirmed by the effect of denaturation of 6 M guanidine hydrochloride on the fluorescence decay. For both conjugates, the longest lifetime is reduced to the same value (ca. 60 nsec), which is considerably smaller than that for either of the non-denatured conjugates. Since guanidine hydrochloride has hardly any effect on the fluorescence decay of free pyrenebutanol (the decay time of the longer component is increased from ca. 90 nsec to ca. 97 nsec), it can be concluded that this decrease upon denaturation results from an increased interaction of the pyrene with the protein backbone. Consequently, the differences in fluorescence between the non-aged and aged conjugates may be ascribed to stronger interaction between the pyrene moiety and the protein in the aged than in the non-aged conjugate. Since energy transfer from the tryptophans to the pyrene label is about the same for both conjugates (ca. 55%), it is likely that the same interaction site on the protein is involved.

The CPL measurements displayed in Fig. 6 show clearly that the interaction of the excited fluorophore with the protein in the non-aged conjugate, pyrenebutyl-OP(O)(OC_2H_5)Cht, is strikingly different from that in the aged conjugate, pyrenebutyl-OP(O)(OH)Cht. It is also worth noting that the values of the anisotropy factor obtained from the CPL measurements for the excited state differ both in magnitude and sign, for both conjugates, from those obtained from the CD measurements for the ground state.

Table 5: Mean value[a] of fluoreacence decay parametera (±SEM) of the aged and non-aged OP—Cht conjugatea under various conditiona

Pyr-P(O)(OC_2H_5)Cht	α_1	τ_1(na)	α_2	τ_2(na)
buffer (pH = 7.4)	0.13±0.01	103±4	0.018±0.004	12±2
buffer + argon	0.13±0.03	110±4	0.036±0.012	12±7
6 M guanidine-HCl	0.10±0.02	62±3	0.046±0.008	13±2
Pyr-P(O)(OH)Cht				
buffer (pH = 7.4)	0.14±0.01	77±2	0.033±0.009	10±2
buffer + argon	0.12±0.01	81±1	0.045±0.004	10±1
6 M guanidine-HCl	0.10±0.01	62±4	0.042±0.008	16±5

α = amplitude, τ = lifetime, Pyr = pyrenebutyl-O-
a. Results are average of at least three separate measurements.

Information on the accessibility of the pyrene moiety to the external medium in the aged and non-aged conjugates was sought by quenching experiments (2,12). This was done employing positive, negative and neutral quenchers, and caloulating the quenching constants from Stern-Volmer plots. The data thus obtained for quenching of pyrene fluorescence by nitromethane and by cesium and iodide ions are summarized in Table 6.

The data shown are not conclusive with respect to the nature of the quenching of the pyrene fluorescence. The low accessibility of I^- and Cs^+ relative to nitromethane (see K_Q values) is consistent with the presence of a hydrophobic region around the pyrene moiety in both model compounds and the protein conjugates. However, the fact that the quenching of the pyrene in pyrenebutanol is more efficient for all quenchers used when compared to the other model compounds (PBPDC-PBEPF) raises the possiblity that the structure of the phosphoryl moiety itself affects the collisional quenching of the pyrene fluorescence and thereby complicates interpretation of the results obtained for the protein conjugates. Thus, even though the value of K_Q for I^-colliding with the pyrene moiety in the non-aged conjugate is 1.55-fold larger than that

Table 6: Quenching constants (K_Q M^{-1} ± SEM)[a] for quenching of model compounds and protein conjugates by various quenchers, as obtained from Stern-Volmer plots (0.01 M phosphate buffer pH 7.4 at 23±1°C)

Compound or Conjugate	Nitromethane x 10^{-2}	I^- x 10^{-1}	Cs^+ x 10^{-1}
Pyrenebutanol	11.6±2.3	9.4±1.7	3.5±0.2
PBPDC	9.3±0.7	6.8±0.8	1.4±0.2
PBEPF	4.1±1.0[b]	6.8±0.8	1.2±0.2
Pyr[c]P(0)(OC_2H_5)Cht	3.2±0.4	3.1±0.2	0.9±0.1
Pyr-P(0)(OH)Cht	3.1±0.3	2.0±0.2	0.8±0.1

a. = Results are average of at least three separate experiments.

b. = 8.6±0.7 after 24 h at room temperature.

c. = Pyr = pyrenebutyl-O-

obtained for the aged form, while nitromethane and Cs^+ display a constant ratio [K_Q non-aged/K_Q aged] of $\sim$ 1 for both conjugates, these results do not permit a conolusive interpretation. Nevertheless, the difference between I^- and the other two quenchers is clear and indicative of different accessibility of the pyrene moiety in the two conjugates to the negatively charged ion. It should be pointed out that the results for the collisional quenching in the case of Cht differ considerably from those obtained for the corresponding AChE conjugates. For the latter enzyme, nitromethane was found to be a more efficient quencher of the pyrene fluorescence in the non-aged than in the aged conjugate, while I^- displayed the same oollisional quenching constant for both AChE conjugates (2).

CONCLUSIONS

The ^{31}P-nmr spectroscopy results along with the reactivation data strongly support the hypothesis that the aged form of phosphorylated Cht contains a P-O$^-$ bond. Thus, the dihalide pyrenebutyl-O-P(O)Cl_2 is a suitable inhibitor for producing the inhibited aged conjugate. From the fluorescence spectroscopy data, it can be clearly stated that the non-aged conjugate, pyrenebutyl-O-P(O)(OC_2H_5)Cht, differs from the aged form, pyrenebutyl-O-P(O)(OH)Cht. For the latter, the interaction of the fluorophore with the protein backbone appears to be stronger than that for the non-aged species. This difference is most pertinent in considering the physicochemical basis for the known resistance of the aged conjugates to reactivation by nucleophiles.

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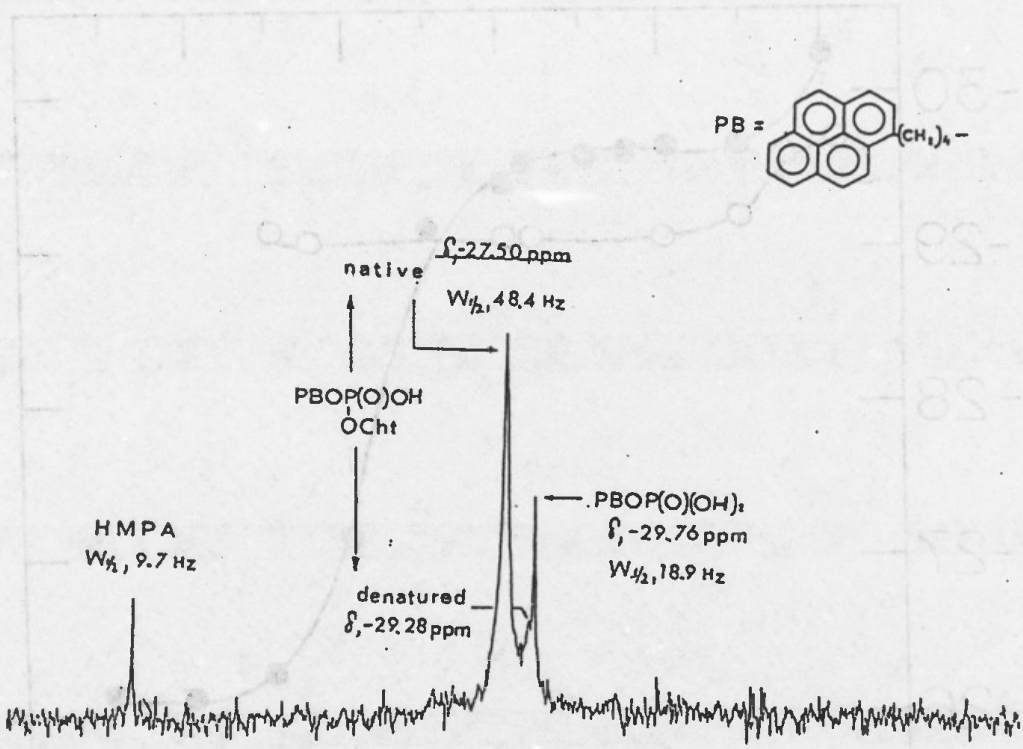

Fig. 1: ^{31}P-nmr spectra of the aged conjugate, pyrenebutyl-O-P(O)(OH)Cht, (1 mM, pH 4.1). Chemical shifts were assigned to HMPA. The diacid signal stems from the corresponding dichloro inhibitor that was not removed completely after the inhibition.

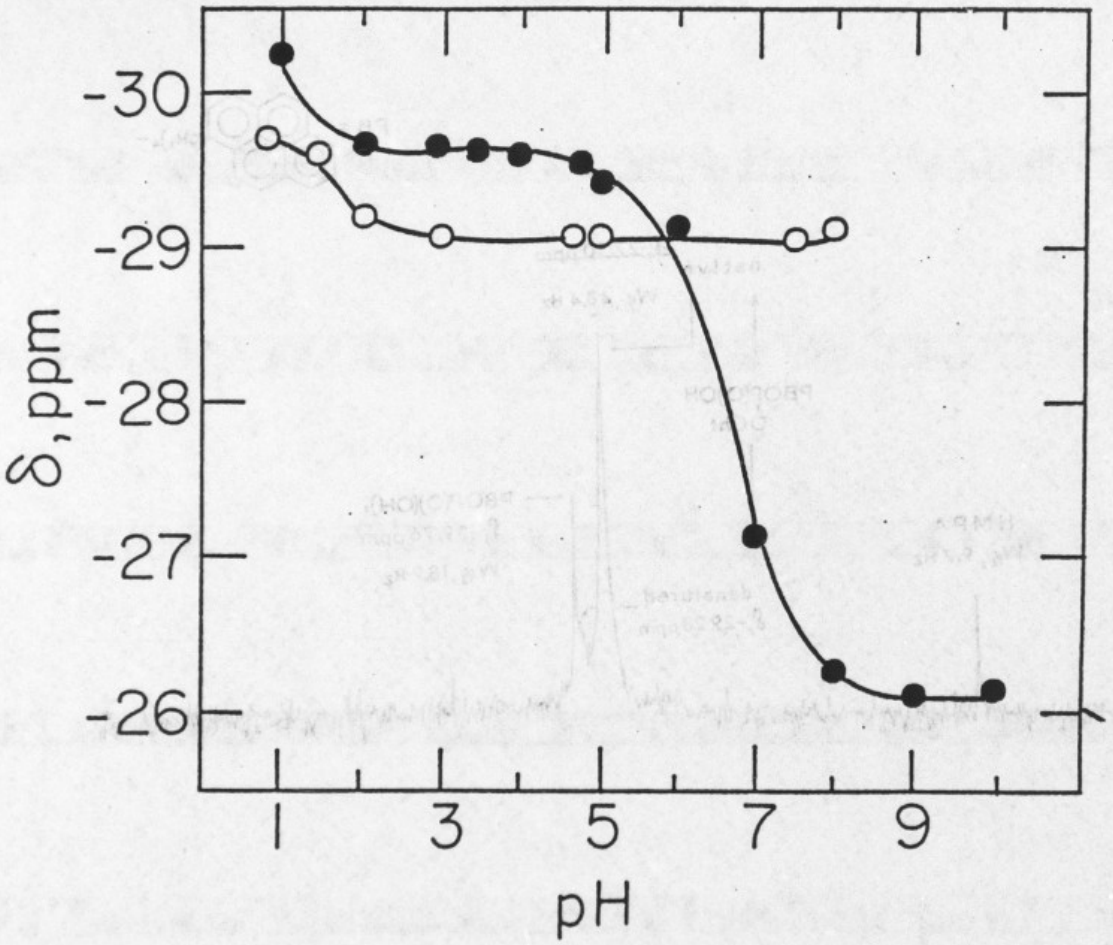

Fig. 2: pH chemical shift profile for mono- and diacid phosphates. δ is related to HMPA. o——o , $(C_2H_5O)_2P(O)OH$; •——• , $(C_2H_5O)P(O)(OH)_2$.

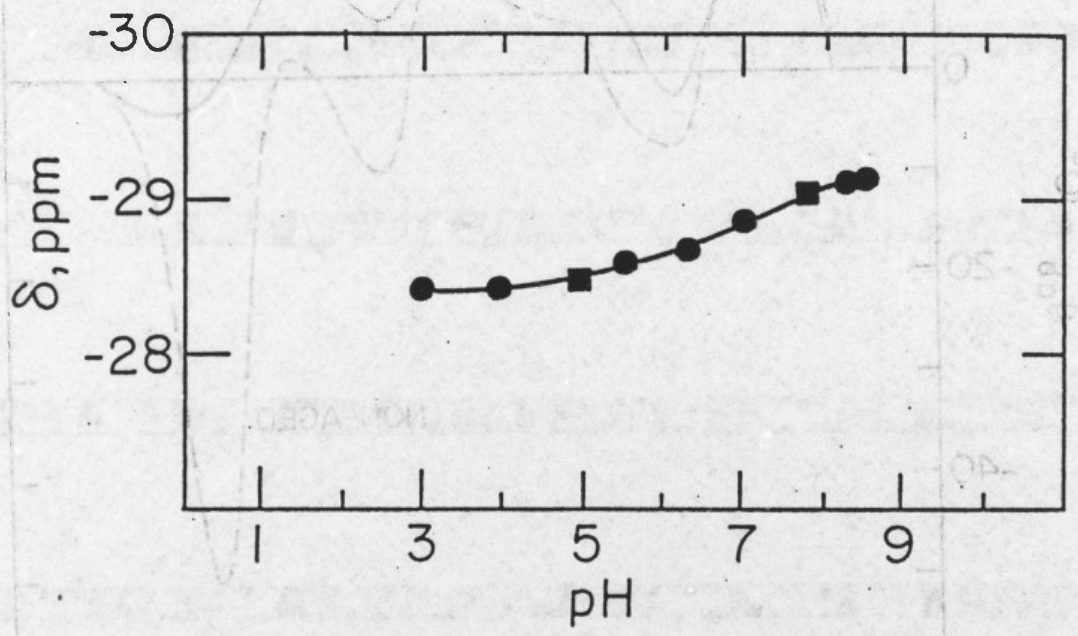

Fig. 3: pH chemical shift profile for $(C_2H_5O)_2P(O)Cht$. δ is related to HMPA. ●——● , conjugate obtained by $(C_2H_5O)_2P(O)F$ ■——■ , conjugate obtained by $(C_2H_5O)_2P(O)O\emptyset NO_2$.

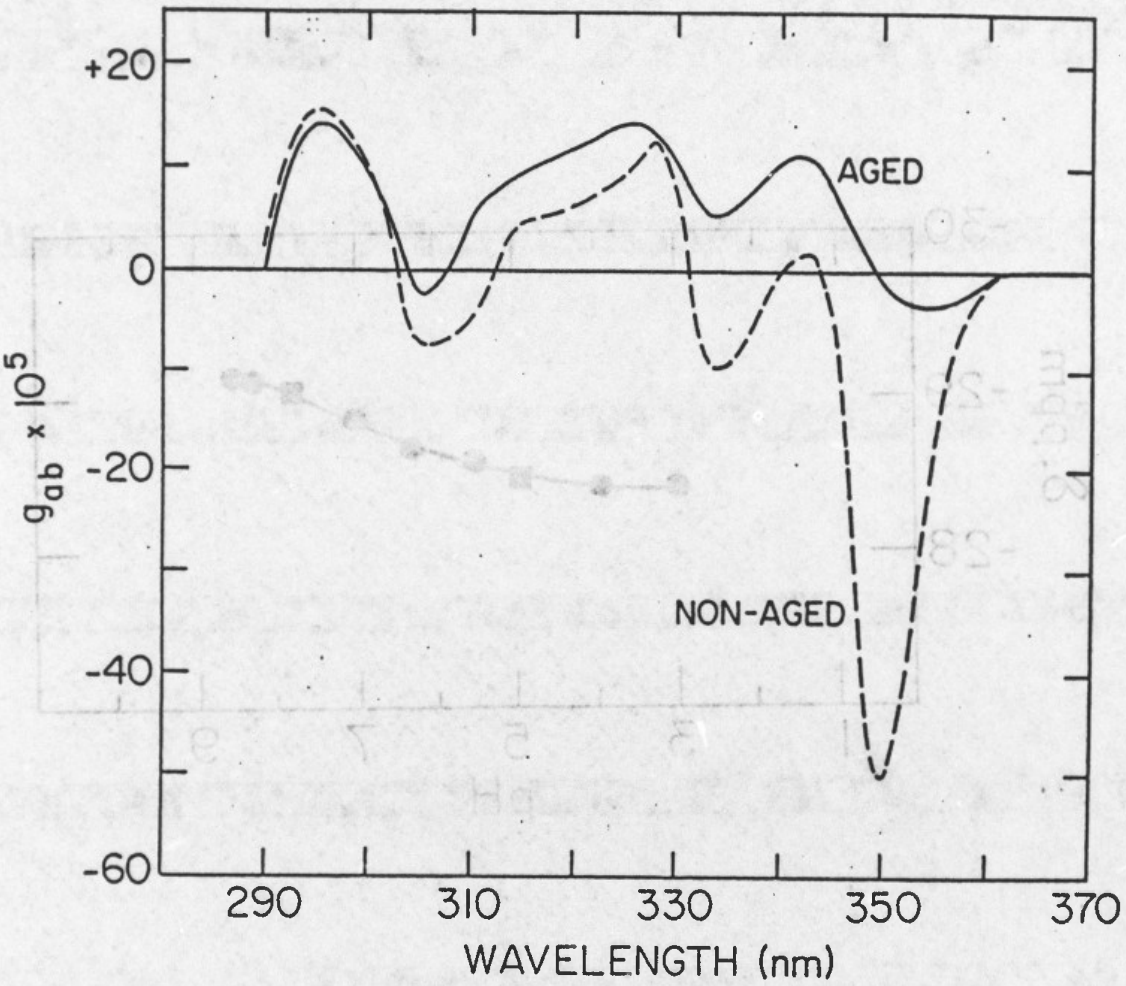

Fig. 4: Circular dichroism (CD) spectra of aged [pyrenebutyl-O-P(O)(OH)Cht] and non-aged [pyrenebutyl-O-P(O)(OC_2H_5)Cht] conjugates of chymotrypsin. The curves display the absorption anisotropy factor, g_{ab}, as a function of wavelength for the aged (——) and non-aged (- - -) conjugates.

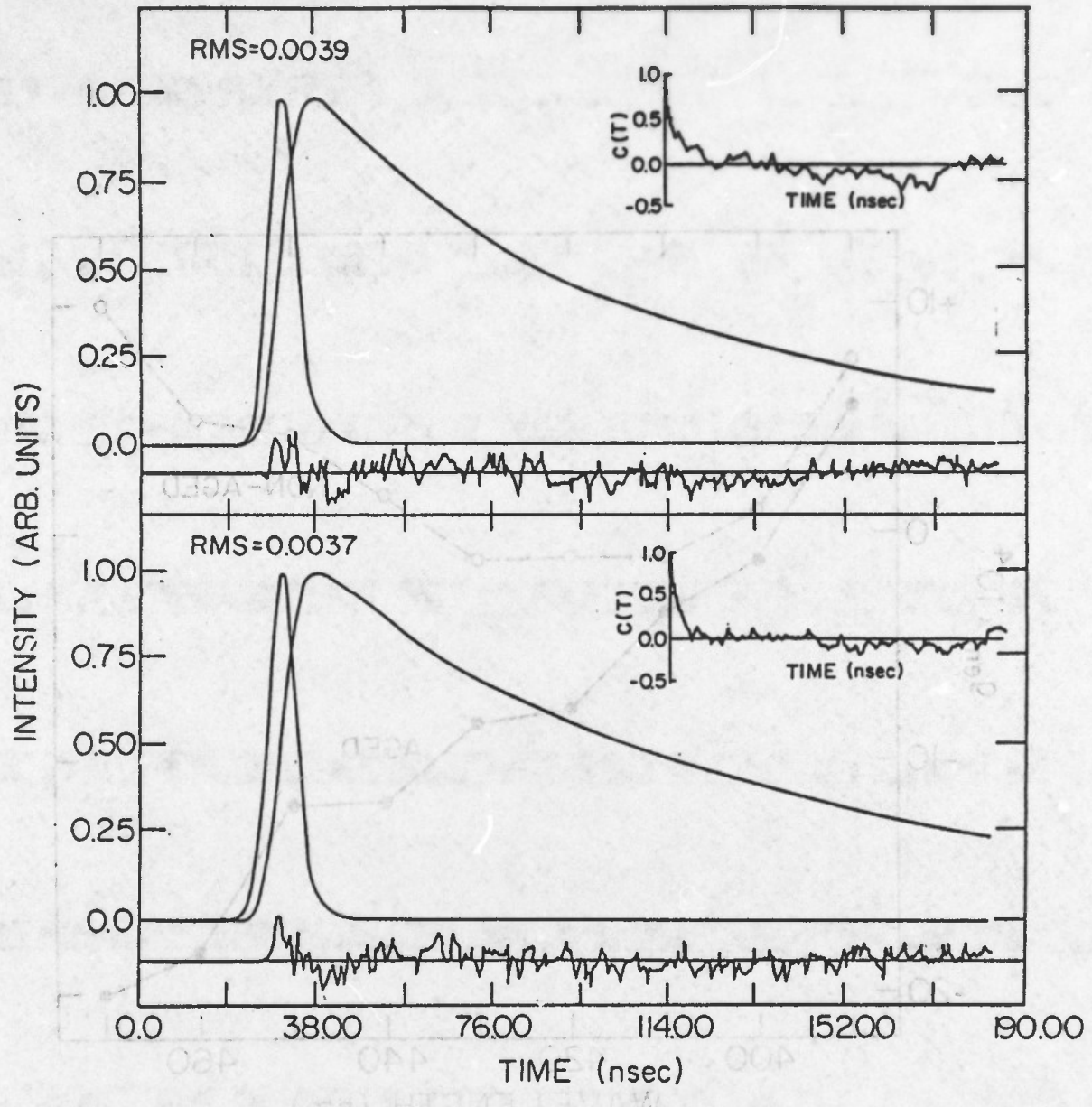

Fig. 5: Fluorescence decay curves of aged [pyrenebutyl-O-P(O)(OH)Cht] (upper) and non-aged [pyrenebutyl-O-P(O)(OC_2H_5)Cht] (lower) conjugates of chymotrypsin. The data were analyzed by assuming a two-component decay curve. Amplitudes and lifetimes obtained were as follows: Non-aged conjugate $\alpha_1 = 0.14$, $\tau_1 = 101.35$ nsec, $\alpha_2 = 0.016$, $\tau_2 = 12.82$ nsec. Aged conjugate $\alpha_1 = 0.13$, $\tau_1 = 79.42$ nsec, $\alpha_2 = 0.025$, $\tau_2 = 12.68$ nsec. The traces of the deviations between the theoretical and experimental decay curves are shown below each curve, and the autocorrelation functions of the deviations are shown in the inset at the upper right.

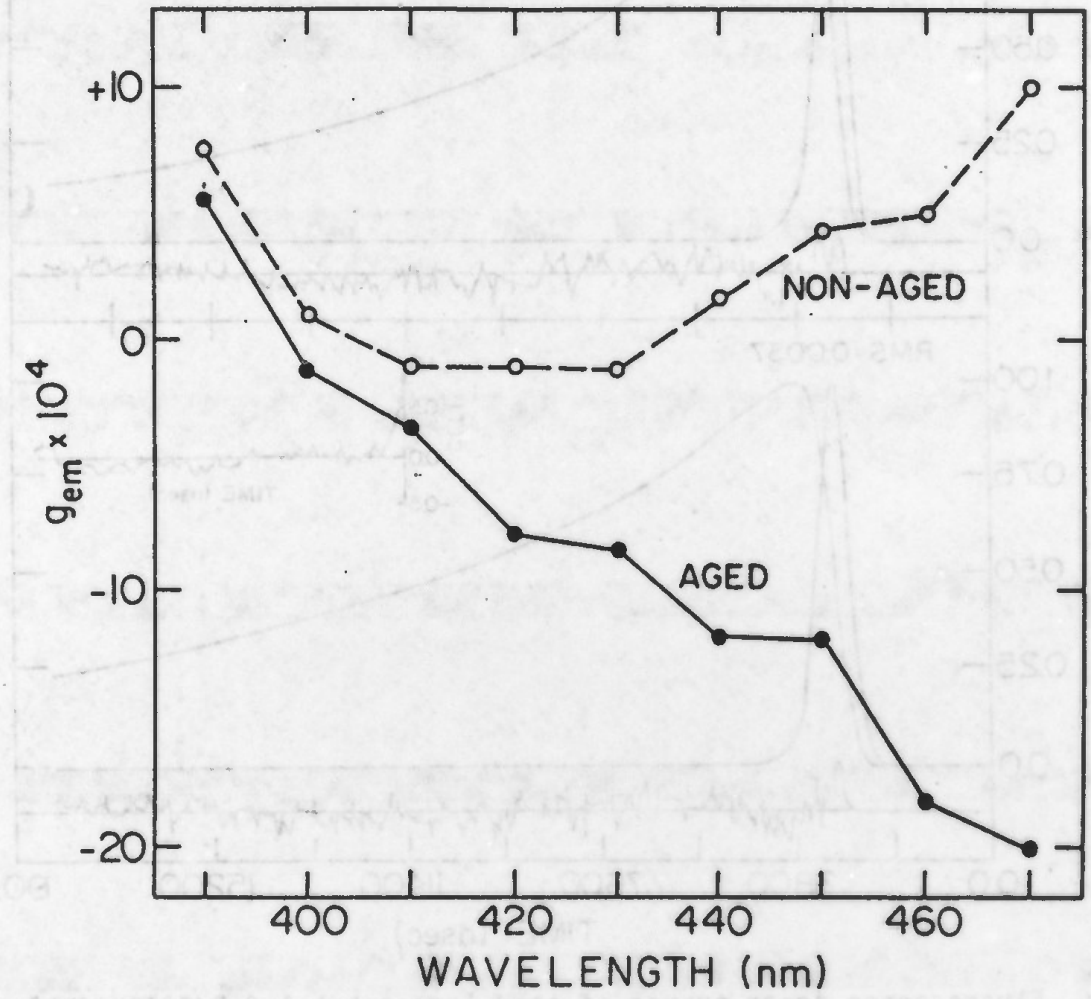

Fig. 6: Circularly polarized luminescence (CPL) spectra of aged [pyrenebutyl-O-P(O)(OH)Cht] and non-aged [pyrenebutyl-O-P(O)(OC_2H_5)Cht] conjugates of chymotrypsin. The curves show the emission anisotropy factor, g_{em}, as a function of wavelength for the aged (●—●) and non-aged (○----○) conjugates.

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